Evaluation of real-time nucleic acid sequence-based amplification for detection of *Chikungunya virus* in clinical samples

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The *Chikungunya virus* (CHIKV) is a member of the genus *Alphavirus* that is transmitted to humans by *Aedes* mosquitoes. In 2005 and 2006, the Indian Ocean island of La Réunion was hit with an unprecedented CHIKV fever outbreak that infected 300 000 people. In the present study, we describe the evaluation of real-time nucleic acid sequence-based amplification (RT-NASBA) for the detection of CHIKV in clinical samples. A co-extracted and co-amplified chimerical CHIKV RNA sequence was used as an internal control to eliminate false-negative results. The detection threshold of the assay was determined from quantified CHIKV-positive plasma, and estimated to be 200 copies per NASBA reaction. The specificity of the assay was determined using BLAST analyses and non-cross-reactivity using an O'nyong-nyong virus culture and 250 CHIKV RT-PCR-negative plasma samples. A 100% specificity was found and no invalid result was obtained, showing the good quality of the nucleic acid extraction. The assay was then evaluated using 252 CHIKV-positive RT-PCR plasma samples. The samples were all tested positive, including those with low viral load. This evaluation showed that the RT-NASBA is a rapid (5 h from sample nucleic acid extraction to detection), sensitive, specific and reliable method for the routine diagnosis of CHIKV in clinical samples.

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INTRODUCTION

Chikungunya virus (CHIKV) is an arthropod-borne virus (arbovirus) and a member of the genus *Alphavirus* in the family *Togaviridae* (Johnston & Peters, 1996). CHIKV is transmitted to humans by mosquitoes belonging to the *Aedes* genus (essentially *Aedes fuscifer* in Africa and *Aedes aegypti* in Asia) (Diallo *et al.*, 1999; Jupp & McIntosh, 1988).

In 2005–2006, *Aedes albopictus* was involved in the CHIKV outbreak in the Indian Ocean. *Ae. albopictus* has recently become established in at least nine countries in Europe, Africa and America (Gratz, 2004) and is disseminated worldwide inside used tyres (Reiter, 1998). Mutation A226V is probably responsible for a significant increase in CHIKV infectivity in *Ae. albopictus* (Schuffenecker *et al.*, 2006; Kumar *et al.*, 2008; Vazeille *et al.*, 2007). First isolated in Tanzania in 1952 (Ross, 1956), CHIKV has caused many outbreaks in Africa (East, Central and West) and Asia (Lam *et al.*, 2001). Since 2005, CHIKV has emerged in several Indian Ocean islands [Comoros, La Réunion (Paquet *et al.*,

2006), Mauritius, Seychelles and Madagascar (Schuffenecker *et al.*, 2006)], in India (Ravi, 2006), and also in Europe, most recently in Italy in 2007 (Angelini *et al.*, 2007; Rezza *et al.*, 2007). Phylogenetic analysis revealed three distinct phylogroups: West African, East Central South African and Asian (Powers *et al.*, 2000). Viral strains of the Indian Ocean 2005–2006 outbreak belonged to the Eastern African phylogroup (Schuffenecker *et al.*, 2006).

Typical clinical signs are polyarthralgia and fever (Borgherini *et al.*, 2007). The positive predictive value of these two signs is excellent during an outbreak (>95%) (Staikowsky *et al.*, 2007). Other symptoms such as asthenia, myalgia, skin rash, and digestive and neurological disorders have been described (Borgherini *et al.*, 2007). Serious forms have also been reported (Lemant *et al.*, 2008). Viraemic parturients represent a substantial risk for neonates (Gérardin *et al.*, 2008a). Asymptomatic forms are rare (<5%) (Gérardin *et al.*, 2008b); this is unlike the case for other arboviruses such as dengue (Endy *et al.*, 2002) and West Nile (Biggerstaff & Petersen, 2003; Gubler, 1989) viruses.

A rapid and accurate method for diagnosis of CHIKV is necessary due to the serious forms and mother-to-child

Abbreviations: IC, internal control; RT-NASBA, real-time nucleic acid sequence-based amplification; RT-PCR, reverse transcription PCR.

transmission, the new repartition of *Aedes* and the recent outbreaks. The commonly used methods in the acute phase are virus isolation from the blood of viraemic patients, which is time-consuming, and serodiagnosis (Beatty *et al.*, 1995). Genomic sequence detection can be used for a rapid diagnosis before the appearance of anti-CHIKV IgM. Conventional RT-PCR methods were recently modified to real-time RT-PCR assays (Carletti *et al.*, 2007; Dash *et al.*, 2007; Edwards *et al.*, 2007; Laurent *et al.*, 2007).

In the present study, a nucleic acid sequence-based amplification (NASBA) technique along with an internal control were developed. The sensitivity and the specificity of the method are described and the value for the diagnosis of CHIKV is discussed.

METHODS

Biological specimens. During the 2005–2006 CHIKV outbreak in La Réunion, the Groupement Hospitalier Sud Réunion Laboratory performed routine diagnosis for patients showing the described CHIKV disease symptoms. Molecular detection and quantification of CHIKV RNA in plasma were done using a TaqMan assay (Laurent *et al.*, 2007) and aliquots of collected plasma were stored at -80°C . A total of 250 RT-PCR-negative and 252 RT-PCR-positive plasma specimens were randomly selected from that collection to assess CHIKV RNA NASBA detection. Viral loads were measured using RT-PCR in 226 of the 252 positive specimens and ranged from 29 100 to 8.55×10^6 genome copies (ml plasma) $^{-1}$.

Sequence alignments. A total of 32 CHIKV E1 gene nucleotide sequences were downloaded from the NCBI sequence database. Alignments were performed using the Vector NTi Advance 10 software (Invitrogen), which includes CLUSTAL 1.8. The CHIKV primers and molecular beacon probe were selected in the most conserved regions of the E1 gene. The positions of the CHIKV primers and molecular beacon on the alignment are represented in Fig. 1. The amplicon generated is a 297 base RNA which is detected in real time by the CHIKV molecular beacon probe. The GenBank accession numbers are also represented in Fig. 1.

NASBA CHIKV internal control (IC). The NASBA CHIKV IC is an RNA transcript that is amplified by the NASBA CHIKV primers and detected by the IC-specific ROX-labelled probe (IC probe). It was constructed from the CHIKV NASBA amplicon by replacing the binding site of the wild-type beacon (FAM) with the IC beacon (ROX) binding region sequence. IC RNA is included in the plasma at 10 000 copies per sample and follows all the process from the nucleic acid extraction to the amplification/detection. Detection of the IC validates the assay; this is important in case potential inhibitors of the amplification are present.

RNA isolation from plasma. Nucleic acid was isolated from clinical samples using the NucliSENS easyMAG platform (bioMérieux) in combination with the NucliSENS magnetic extraction reagents (bioMérieux) and NucliSENS lysis buffer (bioMérieux) according to the manufacturer's instructions. The NucliSENS lysis buffer inactivates CHIKV. Studies showed that it inactivates viruses such as human immunodeficiency virus, bovine viral diarrhoea virus and pseudorabies virus (personal data). Two hundred microlitres of clinical sample (plasma) was added to 2 ml lysis buffer. The mixture was incubated at room temperature for 10 min before and after

Fig. 1. Alignment of the designed primers and probe with CHIKV E1 gene sequences available in GenBank.

addition of a mix containing the magnetic silica and the NASBA CHIKV IC. The mixture was then subjected to the NucliSENS easyMAG platform for nucleic extraction following the off-board protocol according to the manufacturer's instructions. Nucleic acids were eluted in 20 µl elution buffer, and immediately subjected to NASBA amplification.

CHIKV RT-NASBA amplification and detection. A primer mixture was prepared using the reagents from the NucliSENS EasyQ basic kit Version 2 (bioMérieux) and the CHIKV specific primers and probe. CHIKV and IC RNA were co-amplified with the CHIKV primers (CHIKVF: Forward, 5'-GCCTGGACACCTTTCGAC-3'/CHIKVR: Reverse, 5'-AATTCTAATACGACTCACTATAGGGGCTCTTACCG-GGTTTGTTC-3'). The CHIKV amplicons were detected with the CHIKV-specific molecular beacon probe (CHIKV probe, FAM-labelled: 5'-CGAGCGACTCTCAGGCACCATCTGGCTCGCTCG-3') and the IC amplicons by the IC-specific molecular beacon probe (IC probe, ROX-labelled). Amplification reactions were performed using 150 mM KCl. The final concentration in the reaction mixture of each CHIKV primer was 0.4 µM and the final concentration of the FAM and ROX molecular beacon probes was 0.2 µM. For each amplification reaction, 5 µl of extracted nucleic acid solution was added directly to the primers and probes mixture. Removal of secondary RNA structure and primer hybridization was performed by a two-step incubation of 2 min at 65 °C and 2 min at 41 °C. To each reaction, 5 µl enzyme was added, and the RT-NASBA reaction was then performed in the NucliSENS EasyQ analyser (bioMérieux) at 41 °C for 90 min. The fluorescence signal was measured and analysed using the NucliSENS EasyQ Director V2.0 software (bioMérieux). For data analysis, the target detection threshold was set when the positive signal/negative signal ratio reached 1.2.

RT-NASBA detection threshold. The detection threshold of the assay was determined by using 10-fold dilutions of two CHIKV-positive plasma samples and serial 1:2 dilutions of a CHIKV viral culture supernatant. The CHIKV plasma and culture supernatant were previously quantified by TaqMan real-time PCR (Laurent *et al.*, 2007). Dilutions were performed on CHIKV-negative plasma.

RESULTS AND DISCUSSION

In 2005 and 2006, La Réunion was hit with an unprecedented CHIKV fever outbreak. The CHIKV is estimated to have infected about 300 000 people (Perreau *et al.*, 2007) and was responsible for 237 deaths (Reiter *et al.*, 2006). During the outbreak, laboratories had to adapt and develop assays for rapid and reliable CHIKV detection. In the present study, we describe the evaluation of an RT-NASBA assay for the detection of CHIKV. RT-NASBA is a continuous and isothermal reaction. The reaction generates ssRNA amplicons which are detected by a specific molecular beacon. This technology is well adapted for RNA targets especially RNA viruses (Deiman *et al.*, 2007; Capaul & Gorgievski-Hrisoho, 2005).

The specificity of the CHIKV primers and molecular beacon was determined by aligning the sequences against complete arbovirus genome sequences including those of dengue 1 to 4, West Nile virus, tick-borne encephalitis virus and Japanese encephalitis virus. The alignments showed no match of the CHIKV primers and molecular beacon probe with these arbovirus sequences. To confirm

the specificity of the CHIKV primers and molecular beacon probe, the assay was evaluated using different dilutions of an O'nyong-nyong strain. O'nyong-nyong virus is an *Alphavirus* closely related to CHIKV. No positive signal could be generated, even for the highest O'nyong-nyong virus concentrations. Moreover, 250 CHIKV-negative RT-PCR plasma samples obtained from patients admitted to the hospital during the outbreak were tested using the RT-NASBA. No positive signal was detected for all 250 samples. In each case, the IC was successfully extracted, amplified and detected, validating the results. The CHIKV-negative status of the samples was confirmed by RT-PCR. Taken together, the CHIKV RT-NASBA results showed a specificity of 100 %.

The detection threshold of the NASBA assay was determined through three independent experiments, from serial dilutions of two quantified plasma samples and a viral culture. The plasma samples and the viral culture were quantified by TaqMan real-time PCR. The last clear positive signal obtained with the two plasmas was 4180 and 5250 genome copies ml⁻¹, respectively, corresponding to 209 and 263 copies per NASBA reaction. Using the serial dilutions of the CHIKV culture, the last clear positive signal was obtained for the dilution corresponding to 7625 copies ml⁻¹ (381 copies per NASBA reaction). A weak but still visible positive signal for the dilution corresponding to 3813 copies ml⁻¹ (191 copies per NASBA reaction) was observed. The results showed that the assay detected the non-diluted samples and all dilutions down to 200 copies per NASBA reaction, setting the detection threshold for the assay. Considering that CHIKV clinical samples usually have high viral loads (generally above 10⁴ copies ml⁻¹) (Laurent *et al.*, 2007), the assay is sensitive enough to diagnose all CHIKV clinical cases during the viraemic phase, generally less than 8 days after the clinical onset (Laurent *et al.*, 2007). To confirm this, the assay was evaluated with 252 CHIKV-positive plasmas (viral loads ranging from 29 100 copies ml⁻¹ to 8.55 × 10⁶ copies ml⁻¹). All samples were detected with the RT-NASBA assay, showing 100 % sensitivity on these clinical samples. No false-negative signal due to poor nucleic acid extraction or inhibition of the amplification occurred, which indicates the high quality of the nucleic acid extraction system. In order to study potential CHIK false-positive detection, 250 CHIKV-negative plasma samples were tested. There was no positive signal obtained from any of the 250 negative samples. The IC signal was always present, validating all the tests by confirming the absence of inhibition of the amplification. An inhibition of the IC amplification was observed with samples with high viral loads; however, this is not detrimental since the IC is used to monitor false-negative results.

In conclusion, we showed that RT-NASBA can be used as a routine laboratory technique for the detection of CHIKV in plasma samples. In this study, the CHIKV RT-NASBA gave a 100 % positive and negative predictive value. The entire diagnosis, from nucleic acid extraction to detection, can be

performed in 5 h, which is adapted to emergency situations. The 2005–2006 CHIKV outbreaks in the Indian Ocean and the recent cases in Italy (Angelini *et al.*, 2007; Rezza *et al.*, 2007) showed the need for a rapid and reliable method to detect CHIKV.

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